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RELEASE OF ELASTASE FROM PURIFIED HUMAN LUNG MAST CELLS AND BASOPHILS
Identification as a Hageman Factor Cleaving Enzyme[1]

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Abstract—Elastase, a serine protease, is capable of inducing severe lung destruction in experimental animal models. We now report that this proteinase exists preformed in neutrophil-free sonicates of purified human lung mast cells (>98% purity) and in circulating peripheral blood basophils (>97% purity). The elastase levels in both cell types (41–174 ng/10^6 cells) represents approximately 3–20% of those found in human neutrophils; both cell types released their elastase following anti-IgE and ionophore A23187 challenge. The apparent molecular size of the mast cell enzyme on Sephadex G-100 gel filtration, as well as its inhibition profile, was identical to that of purified human neutrophil elastase. This mast cell elastase is identical to our previously reported mast cell-derived Hageman factor cleaving activity. Mast cell-, basophil-, and neutrophil-derived elastases cleave Hageman factor into fragments of 52,000 and 28,000 Da; cleavage by all three enzymes is inhibited by preincubation with polyclonal antibodies directed against human neutrophil elastase.

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INTRODUCTION

Despite the identification of a growing number of mediators released from pertubed tissue mast cells and peripheral blood basophils, the precise role of many mediators in the pathophysiology of the allergic process remains poorly defined (1–4). Also. many aspects of basophil and mast cell-mediated reactions lack explanation based on known mediator activity (4). Perhaps the least understood mediators are those with proteolytic activity (5–12). On a per cell basis, tryptase is the predominant protease of the human mast cell (10), although only small amounts are present in basophils (13). Although activities have been described for this enzyme *in vitro*, its relevant *in vivo* effect remains speculative. More recently, we and others have defined a mast cell enzyme(s) with chymotryptic specificities (11, 12, 14); however, its role remains conjectural.

With the discovery of a TAMe (tosyl arginine methyl ester) esterase activity (5), we began to investigate the release of other high-molecular-weight enzymes that may act as inflammatory mediators in IgE-dependent reactions. We reported the release, following antigen challenge of sensitized human lung fragments, of four additional proteases separated on the basis of QAE Sephadex (6–9) that interact with components of the Hageman factor (HF) -dependent pathways. These enzymes include lung Hageman factor cleaving factor (LHFCF) (6, 7), a lung prekallikrein activator, and two lung kininogenases (8).

In a preliminary communication on the characterization of the LHFCF, we observed that human neutrophil elastase produced a Hageman factor cleavage pattern that was identical to the one generated by the mast cell-derived enzyme (11); this finding led to our current detailed studies. We now confirm that LHFCF is a preformed protease contained in neutrophil-free preparations of both human lung mast cells and circulating basophils; it is secreted from the cytoplasmic granule, along with histamine, in an IgE-dependent manner. We now also present evidence based on apparent molecular weight, substrate cleavage profile, multiple antibody inhibition profiles, and antigenicity to indicate that LHFCF is a mast cell and basophil elastase. To date, LHFCF cannot be distinguished from human neutrophil elastase.

MATERIALS AND METHODS

The following materials were purchased: diisopropyl-fluorophosphate (DFP) (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin); chromatographic gels and equipment (Pharmacia Fine Chemicals, Piscataway, New Jersey); slab gel electrophoresis chemicals and apparatus (Bio-Rad Labs., Richmond, California); Pronase and chymopapain (Sigma Chemical Co., St. Louis, Missouri); bovine serum albumin (Reheis Chemical Co., Chicago, Illinois); DNase, collagenase, and

porcine pancreas elastase (Cal Biochem, LaJolla, California); X-ray film and holder (Eastman Kodak, Rochester, New York); radiolabeling and basic laboratory chemicals (Fisher Scientific Co., Pittsburgh, Pennsylvania); and [125]NaI (New England Nuclear, Boston, Massachusetts). We were kindly provided with ragweed antigen E (AgE), C1 inhibitor (NIAID, NIH, Bethesda, Maryland), alpha-1-antitrypsin (Dr. R.W. Berninger, Tufts Medical School, Boston, Massachusetts), and affinity purified anti-IgE (Dr. N.F. Adkinson, Jr., Johns Hopkins School of Medicine, Baltimore, Maryland).

Preparation of Hageman Factor. Hageman factor was purified from human plasma by fractionation on both ion-exchange and affinity chromatography as previously reported (15, 16). The purified Hageman factor was then radiolabeled using the chloramine T method (17).

Preparation of LHFCF. LHFCF was isolated free of other known proteases as previously reported, by fractionation of anti-IgE-generated human lung supernatants on DEAE-Sephadex, SP-Sephadex, and G-100 Sephadex (7, 8, 11).

Hageman Factor Cleaving Assay. The assay was performed as previously described (18). Twenty microliters of a 0.01 M Tris-buffered saline (pH 7.4, TBS) solution containing 1 mg/ml bovine serum albumin (BSA) and 10 μl of [^{125} I] HF (10 pg/ml)(0.05 μCi/ml) were placed in a glass tube and shaken at 60 oscillations per minute for 5 min at room temperature. The liquid was removed, and the tube was washed twice with 200 μl of TBS to remove all unbound HF. Ten microliters of a solution containing 1 mg/ml BSA in 0.01 M Tris-0.5 M sodium chloride (pH 7.4) were then added to the glass tube containing the bound HF. After shaking the tube for another 2 min, 20 μl of the test sample were added and the shaking continued for another 60 min at room temperature. Ten microliters of a solution of 20% SDS and 4% mercaptoethanol were then added and the tube was placed in boiling water for 4 min. The contents were electrophoresed using the Laemmli gel system with a 9% acrylamide SDS slab gel (19), fixed, and stained with a 2.5% Coomassie blue R-250 solution in a 1 : 1.5 : 7.5 mixture of methanol–acetic acid–water. After destaining, the gels were dried and exposed to Kodak X-Omat film at −70°C. The film was developed, and the amount of cleavage was either subjectively graded on a scale of one to four (where four represented complete cleavage of the 80,000 mol wt zymogen HF) or scanned by a DU-8 Beckman spectrophotometer. Inhibition of LHFCF-induced breakdown of Hageman factor was determined by adding 20 μl of different protease inhibitors to the 20 μl of LHFCF, then the assay was performed as indicated above.

Isolation of Mast Cells and Basophils. Human lung mast cells were isolated and purified as previously described (20–22). Briefly, macroscopically normal human lung tissue was recovered from surgical specimens excised from patients with lung carcinoma. The tissue was dissected free of pleura, cartilage, and large vessels, and minced. The resulting fragments were twice incubated with Pronase and chymopapain and twice incubated with porcine pancreatic elastase and clostridial collagenase. The freed cells were then subjected to countercurrent elutriation centrifugation followed by either density gradient centrifugation (23) or affinity chromatography (24). Human basophils were isolated from the blood of normal individuals by gradient centrifugation and affinity chromatography (24). Wright- Giemsa-stained cytocentrifuge preparations in which at least 1000 cells were counted failed to show neutrophil contamination of either mast cell or basophil preparations. In release experiments, purified basophils and mast cells were challenged with either affinity purified anti-IgE, ionophore A23187, or buffers at 37°C for 20 min (mast cells) or 45 min (basophils).

Purification of Human Neutrophil Elastase (HNE). Neutrophils were isolated from the peripheral blood of normal donors by standard dextran sedimentation and Ficoll-Hypaque techniques (25). The pellet fraction, containing greater than 93% neutrophils, was extracted in 1.0 M NaCl containing 0.05 M Tris HCl, pH 8.0. HNE was purified 90-fold by a modification of the method of Travis et al. (26), utilizing sequential Aprotonin-Sepharose affinity chromatography, CM-cellulose ion-exchange chromatography, and AcA_{44} gel filtration chromatography (27). Sodium

dodecyl sulfate-polyacrylamide gradient gel electrophoresis of the reduced purified protein demonstrated three polypeptides of M_r 31,000, 28,000 and 27,500. However, analysis of the leading peak of the elution profile using AcA_{44} gel filtration chromatography gave an apparent molecular weight of 12,000. The discrepancy between these molecular weight determinations may be related to physical interaction of the enzyme with the gel matrix, possibly by hydrophobic interactions (27).

Solid-Phase Radioimmunoassay for HNE. A monospecific, precipitating antibody capable of inhibiting elastinolysis by HNE was produced by repeated immunizations of a goat. The IgG fraction and the affinity purified antibodies were used to develop a recently described solid phase radioimmunoassay for HNE (28) with sensitivity in the range of 0.08–4 ng/ml. There was no cross-reactivity in binding of the radiolabeled antisera with lactoferrin, cathepsin G, or several serine proteinases including porcine pancreatic elastase with similar amino terminal amino acid sequences (28).

Histamine Release. After challenge of basophils or mast cells with release stimuli or buffer, cells were pelleted and the supernatant removed. Samples were assayed for histamine content by the automated fluorometric assay of Technicon (Tarrytown, New York). Total cellular histamine was determined by lysing the cell pellets in 2% percholoric acid. Histamine release into the supernatant was expressed as a percentage of the total present in both supernatant and lysed pellet.

RESULTS

Presence of LHFCF Activity in Mast Cell and Basophil Preparations. Since release of LHFCF from human lung fragments is IgE-dependent (6, 7), we examined purified human mast cells and basophils to determine whether these two cells (which bind IgE with high affinity), contained the cleaving activity. Human lung mast cells (>98% pure) were placed in Tyrode's buffer at a concentration of 1×10^6 cells/ml, and sonicated four times × 30 sec using a Branson sonicator (Branson Inc., Danbury, Connecticut) with a microprobe at 4°C. The resultant suspensions were centrifuged at 900*g* for 15 min at 4°C, the supernatants incubated with [^{125}I] HF, and the mixture tested for Hageman factor cleaving activity. Supernatants derived from mast cells completely cleaved the Hageman factor (Figure 1A), as noted by the depletion of radiolabeled zymogen. The TBS control (also incubated for 1 h) had a major band at molecular weight 80,000 Da, representing native HF; cleavage products formed by autoactivation (18) were also present as indicated by the 52,000- and 28,000-Da bands. This experiment was repeated using similarly sonicated basophils (97% pure) at a concentration of 2×10^6 cells/ml. As shown in Figure 1*b*, basophils also possessed a preformed factor capable of cleaving Hageman factor.

IgE-Dependent Release of LHFCF. Mast cells were challenged at 37°C for 20 min with either buffer alone or affinity purified goat anti-IgE (1 μg/ml) then centrifuged at 900*g* for 15 min at 4°C. Histamine release was less than 5% of total for cells incubated in buffer and 46% of total for cells incubated in anti-IgE. The IgE dependence of the extracellular release of LHFCF is shown in Figure 2. When the LHFCF contents of the supernatants were compared, the cells incubated with anti-IgE released sixfold greater LHFCF than buffer-chal-

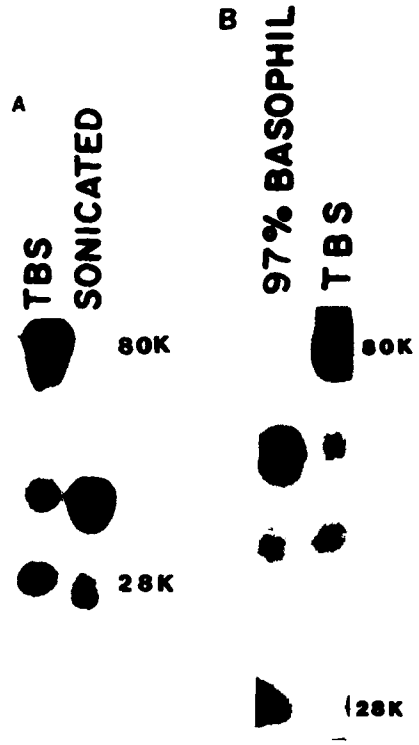


Fig. 1a. (A) Autoradiogram of a reduced SDS gel demonstrating cleavage of [^{125}I]HF by TBS control (left) and by sonicated purified mast cell supernatant (right). Activated HF is seen as bands at molecular weight 52,000 and 28,000. Complete HF cleavage results after incubation with the mast cell sonicate. This figure is representative of four purified and sonicated mast cell preparations. (B) Autoradiogram patterns demonstrating that sonicated basophil (97% pure) supernatant completely cleaves [^{125}I]HF. Two separate basophil preparations demonstrated similar HF cleavage patterns.

lenged cells (as determined by diluting the anti-IgE supernatant until it demonstrated similar activity to the buffer control). In separate experiments, the addition of our affinity purified goat anti-IgE antibody to supernatants from buffer-challenged mast cells failed to enhance cleavage of HF over buffer alone.

Comparison of Elastase and LHFCF. The physical properties of LHFCF (molecular weight 13,000 on G-100 Sephadex) derived from human lung mast cells and its inhibition profile suggested similarities to HNE (28). We therefore examined, in our assay, the cleavage of HF by purified HNE (0.025–25 ng/ml) and compared these results to mast cell- and basophil-derived LHFCF. As shown in Figure 3, the pattern of HF cleavage by HNE was typical to that seen with LHFCF and basophil enzyme. Both HNE and the mast cell-derived enzyme cleaved the 80,000 mol wt zymogen into a 52,000- and a 28,000-mol wt frag-

Fig. 2. Autoradiogram patterns demonstrating IgE-dependent mast cell release of an enzyme inducing cleavage of [^{125}I]HF. In two other mast cell preparations, anti-IgE released four- and eightfold greater LHFCF than the buffer control.

ment. HNE, mast cell, and basophil enzymes could further digest the 52,000-mol wt fragment to one of 40,000 mol wt.

LHFCF, fractionated on Sephacryl S-200, eluted as a globular protein of approximately 12,000–13,000 molecular weight. These findings showed a similarity to purified HNE which fractionated on AcA_{44} at a molecular weight of 12,000 (27). The disparity between the latter molecular weight and that of 27,500–31,000 observed for neutrophil elastase using SDS electrophoresis suggests an interaction of the enzyme with the gel matrix, possibly by hydrophobic interactions (27). These enzymes shared enzymatic characteristics in the HF cleaving assay. All activities were destroyed by heating at 100°C for 15 min or at 60°C for 60 min, and their proteolytic activities were enhanced by increasing the sodium chloride concentration to 1.0 M. All enzymes had a pH optimum

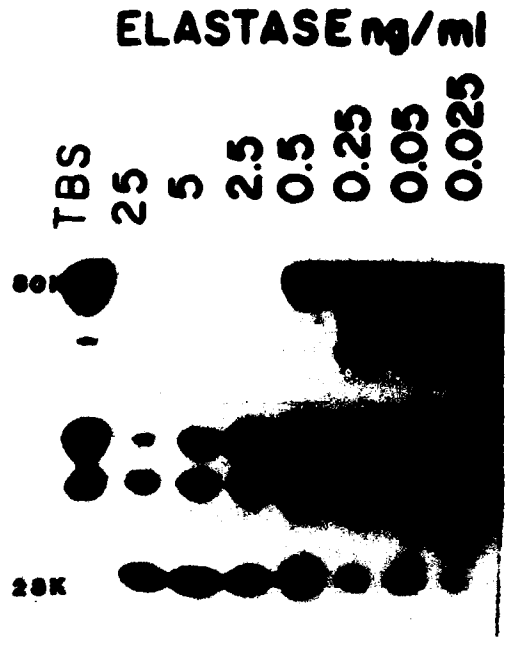


Fig. 3. Autoradiogram demonstrating cleavage of [^{125}I]HF by serial dilutions of purified human neutrophil elastase.

between 6.8 and 7.4. Purified mast cell sonicates and HNE also demonstrated the same pattern of inhibition by protease inhibitors (Table 1). In these experiments, 20 μl of elastase or LHFCF at a concentration found to totally cleave the 80,000-mol wt HF zymogen, were preexposed to the indicated concentrations of the inhibitor. Samples were incubated for 15 min at room temperature before assaying for HF cleaving activity. Inhibitors were classified as effective if they prevented cleavage of the zymogen such that there was no difference between the buffer control and the inhibited enzymes. If HF cleavage was inhibited up to 50%, the inhibitor was classified as a weak inhibitor. If >50% of the zymogen molecule was cleaved, the inhibitor was assumed to be ineffective. Effective inhibitors of both enzymes included diisopropylfluorophosphate, Trasylol, alpha-1-antitrypsin, and soybean trypsin inhibitor. Weak inhibitors were lima bean trypsin inhibitor ($\leq$1 mg/ml), and C1 inhibitor (0.1 mg/ml). Benzamidine ($\leq 5 \times 10^{-2}$ M, an inhibitor of all known Hageman factor activators except LHFCF), epsilon amino caproic acid ($\leq 10^{-1}$ M, an inhibitor of plasmin), cysteine ($\leq 10^{-4}$ M, an inhibitor of sulfhydryl-containing enzymes), and EDTA ($\leq 10^{-1}$ M, an inhibitor of metalloenzymes) failed to inhibit both enzymes. As a control for nonspecific effects due to added protein, BSA and

Table 1. Comparison of LHFCF and Neutrophil Elastase

	LHFCF	Elastase
Mol. size by gel filtration	13,000	12,000
Enhanced activity by NaCl[a]	+	+
pH optimum[a]	6.8–7.2	6.8–7.2
Inhibitors[a]		
DFP	(10^{-3} M)	+
Trasylol	(10^{-1}mg/ml)	+
Alpha-1-antitrypsin	(10^{-1}mg/ml)	+
Soybean trypsin inhibitor	(10^{-1}mg/ml)	+
Activity blocked by IgG from		
Antisera to human elastase[a]	+	+
Preimmune serum[a]	–	–

[a] Measured as an effect on HF-cleaving activity.

HSA at concentrations $\leq$20 mg/ml, failed to have any inhibitory effect on HF cleaving.

Immunological Identify of LHFCF and HNE. Since elastase and LHFCF shared a wide spectrum of physicochemical characteristics, we examined the possibility that the enzymes also shared common immunologic determinants. We examined the capacity of a monospecific antibody to HNE to inhibit the HF-cleaving activity of HNE-, mast cell-, and basophil derived-enzymes. Before addition to the HF cleaving assay, 20 μl of anti-HNE were incubated for 10 min at room temperature with either 20 μl of elastase (20 ng/ml), 20 μl of mast cell supernatant [from 1×10^6/ml mast cells (97% pure) challenged with anti-IgE], or 20 μl of basophil supernatant [from 2.2×10^6/ml basophils (60% pure) challenged with anti-IgE). As shown in Figure 4A and 4B, the anti-elastase antibody blocked the cleavage of HF by all enzyme preparations. Control sera from normal preimmune goats (adjusted for protein concentration) did not affect the HF cleaving activity of these enzymes. In two additional experiments, a second monospecific antibody also neutralized mast cell supernatant and HNE HF cleaving activity.

Quantitation of Elastase Activity in Mast Cells and Basophils. The concentration of elastase in both mast cells and basophils was quantified by an extremely sensitive radioimmunoassay for human neutrophil elastase (28). Two neutrophil-free preparations of mast cells (the first preparation contained 3×10^5 cells/ml of >95% purity and the second contained 10^6 cells/ml of >93% purity) were sonicated and examined for their elastase content. Mast cells from these preparations had elastase concentrations of 40.8 ng/10^6 cells and 58.8 ng/10^6 cells, respectively, which is approximately 3–5% of the elastase we have found in neutrophils (28). When three neutrophil-free mast cell preparations

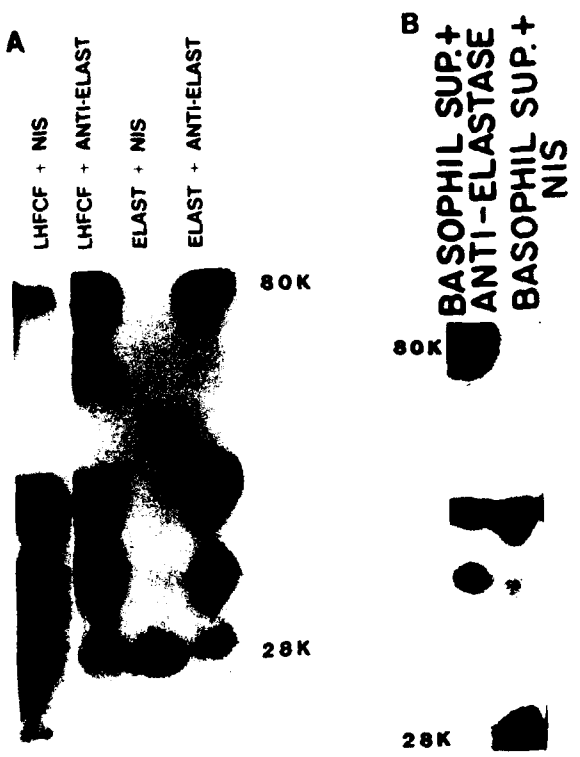


Fig. 4. Autoradiogram demonstrating that cleavage of [^{125}I]HF by both the mast cell enzyme (LHFCF) and the neutrophil enzyme (elast, 25 ng/ml) is inhibited by monospecific antibody to elastase (anti-elast). The preimmune IgG fraction of goat serum (NIS) failed to block cleavage of [^{125}I]HF. This figure is representative of similar results obtained with three other mast cell supernatants. (b) Autoradiogram demonstrating that basophil enzymatic activity was similarly inhibited by a monospecific antibody to elastase but not by preimmune IgG (NIS).

(between 77 and 94% pure) were challenged with ionophore A23187 or anti-IgE, they released 20–53% and 16–46%, respectively, of their total elastase content.

A neutrophil-free basophil preparation (54% pure) contained 100 ng of elastase/10^6 cells or 174 ng of elastase/10^6 basophils. Purified lymphocytes, which represented the major contaminant in the basophil preparations, had no detectable levels of HNE.

To further evaluate the possibility of neutrophil contamination of cell preparations, the presence of the neutrophil-specific granule marker lactoferrin was measured by a radioimmunoassay. On a weight basis, this enzyme would be expected to be found at 10-fold greater concentrations than HNE (Louis Heck,

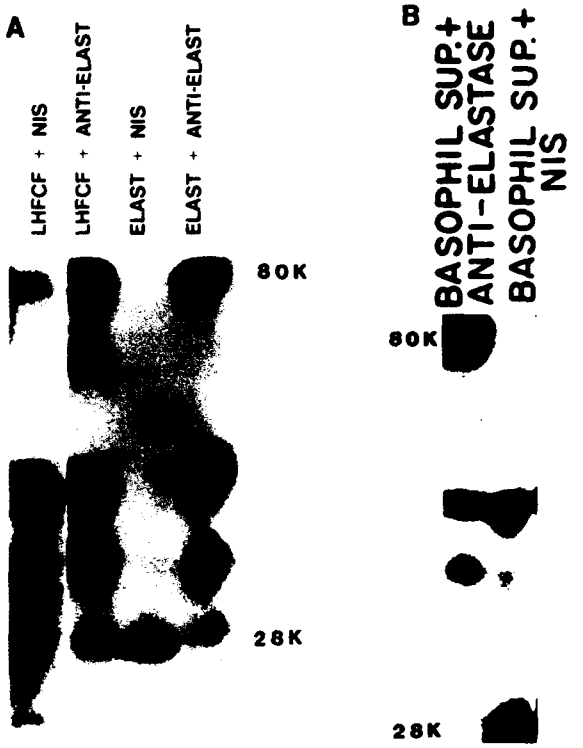
unpublished observation). The basophils contained >0.7% of the lactoferrin expected if the elastase content was attributable to contaminating neutrophils. Mast cells demonstrated even less: <0.1% of the lactoferrin expected if the source of the elastase was the neutrophil.

DISCUSSION

Elastase, a serine protease, has been extensively studied as a crucial mediator of inflammatory lung injury (29–31). Its causative role in emphysema is felt to be amplified by a relative lack of, or impotence of, endogenous lung protease inhibitors (29, 30). The extracellular release of this enzyme during states of active inflammation is presumed to originate from stores in neutrophil azurophilic granules (32). Another potential source of the neutrophil protease is indirect: exocytosed neutrophil enzyme can be pinocytosed and subsequently rereleased by alveolar macrophages (33).

We have now shown detailed evidence that the two principal cell types of human hypersensitivity, the human mast cell and basophil, both contain and release a Hageman factor cleaving activity, and the enzyme responsible is functionally, physically, and immunologically indistinguishable from HNE. These data confirm our preliminary observations (11). Our techniques to purify large numbers of mast cells (21) have allowed more complete comparative studies on the mast cell enzyme with HNE than were possible with the basophil enzyme. The mast cell elastase appears identical to HNE by the following criteria: molecular weight, Hageman factor cleavage products, inhibition profile, synthetic substrate activity, and inhibition by monospecific antibodies. The enzyme is preformed (i.e., stored in granules) and released by cell secretory mechanisms. Secretion can take the form of both an immunological stimulus, such as anti-IgE, and a nonimmunological stimulus, such as calcium ionophore A23187. The possibility of neutrophil contamination in our purified cell preparations has been excluded by three criteria: (1) direct and exhaustive visual examination of stained smears failed to disclose any neutrophils, (2) failure to detect the specific granule marker protein, lactoferrin, and (3) in the case of the mast cell, the known inability of short-lived neutrophils to survive the 72–96 h of culture required for generating pure-cell preparations. Recently there has been verification of our basic findings using immunohistochemical and immunolocalization techniques. Dr. Aaron Janoff (personal communication) has found elastase in human basophils using immunofluorescent antibody techniques. Also, Damiano et al. (34), using immunogold labeling have localized elastase to the human mast cell granule by electron microscopy.

The release of elastase by both mast cells and basophils, the principal cell



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The release of elastase by both mast cells and basophils, the principal cell

types of hypersensitivity, suggests a previously unsuspected role for this protease in allergic disorders. To date, only two other human lung mast cell proteases have been characterized in detail. Tryptase, is the major neutral serine protease of the human mast cell (10). We and others have identified an enzyme(s) with chymotryptic specificities in human lung mast cells (11, 12, 14, 35). Both enzymes exist preformed and are released in parallel with histamine, suggesting a functional localization to mast cell granules. Recent work by Schwartz and coworkers (14, 35) has demonstrated that while all mast cells contain the former enzyme, only 10% of lung mast cells and 90% of skin mast cells contain the latter enzyme. Recently, using an immunoperoxidase procedure with anti-tryptase antibodies, that same laboratory demonstrated that the tryptase was present in only a subpopulation of basophils (13). Similar studies using antibodies to elastase could aid in the identification of elastase-enriched subpopulations of basophils or mast cells. The measurements of elastase using our radioimmunoassay (40–170 ng/10^6 cells) assumes that all mast cells and basophils contain this protease. This per cell figure could be higher if select elastase-rich subpopulations within anatomic sites or specific organs were to be identified. The apparent higher levels of elastase in basophils vs. mast cells may only reflect limited sample numbers. Damiano et al. (34), using an ELISA assay for HNE, reported a range between 30 and 150 ng/10^6 mast cells. The latter value is similar to that found in the basophil.

At present we do not know if the human mast cell biosynthesizes elastase and/or traps HNE by vesicular transport (36). Even if the latter were the case, under the proper conditions of pathophysiology, stored elastase would be released into the mast cell microenvironment. The anatomical localization of large numbers of mast cells in the lung interstitium, together with their long life (months to years), puts these cells in a prime position to mediate interstitial lung injury. This novel role for mast cells would contrast with the traditional concepts of the mast cell solely mediating lung hypersensitivity reactions. Along these lines, numerous recent observations in both animal and human models found that fibrotic lung diseases and articular diseases including rheumatoid arthritis are associated with enormous increases in mast cell numbers and their activation (37–43).

Because of the limited ability to assay low levels of elastase activity by conventional elastinolysis and amide assays, elastase levels have only recently been shown to be elevated in human pulmonary disorders. Although the HF cleaving assay has been used to demonstrate the presence of functional elastase in lavage fluid of patients with acute respiratory distress syndrome (33), we have shown a sensitivity for this functional assay to levels as low as 25 pg/ml. This contrasts with the immunologic elastase assay, which has a sensitivity of 1 ng/ml. The recent availability of similarly sensitive radioimmunoassays will prove more practical for these analyses.

In summary, the release of elastase from the human mast cell and basophil suggests that this protease may play a role in immediate hypersensitivity reactions. Since the mast cell is localized to connective tissues, the role of this enzyme in other types of inflammatory tissue injury, especially under conditions of low anti-protease activity, will require further examination. Furthermore, the role of the mast cell enzyme in human disorders may hinge upon the relative concentrations of the enzyme in mast cells of a particular subtype or anatomic location. Hageman factor cleavage by elastase probably represents only a minor function compared to its potential protean effects on connective tissue substrates.

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Abstract—Elastase, a serine protease, is capable of inducing severe lung destruction in experimental animal models. We now report that this proteinase exists preformed in neutrophil-free sonicates of purified human lung mast cells (>98% purity) and in circulating peripheral blood basophils (>97% purity). The elastase levels in both cell types (41–174 ng/10^6 cells) represents approximately 3–20% of those found in human neutrophils; both cell types released their elastase following anti-IgE and ionophore A23187 challenge. The apparent molecular size of the mast cell enzyme on Sephadex G-100 gel filtration, as well as its inhibition profile, was identical to that of purified human neutrophil elastase. This mast cell elastase is identical to our previously reported mast cell-derived Hageman factor cleaving activity. Mast cell-, basophil-, and neutrophil-derived elastases cleave Hageman factor into fragments of 52,000 and 28,000 Da; cleavage by all three enzymes is inhibited by preincubation with polyclonal antibodies directed against human neutrophil elastase.

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